AWARD NUMBER: W81XWH-13-1-0300

TITLE: Myofibroblast Inhibition to Prevent Posttraumatic Joint Contracture

PRINCIPAL INVESTIGATOR: Sandeep Kathju, MD, PhD

CONTRACTING ORGANIZATION: University of Pittsburgh
Pittsburgh, PA 15213

REPORT DATE: September 2014

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
September 2014	Annual Report	1 Sep 2013 - 31 Aug 2014

4. TITLE AND SUBTITLE

Myofibroblast Inhibition to Prevent Posttraumatic Joint Contracture

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-1-0300

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Sandeep Kathju, MD, PhD

E-Mail: kathjus@upmc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Pittsburgh
Pittsburgh, PA 15213

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Fibrosis in the joints of the extremities is due to the action of myofibroblasts, cells activated by the inflammatory cascade of injury to effect scarring and contracture. There is as yet no medical or pharmaceutical adjuvant therapy that can prevent or treat scar contracture in the joint. We postulate that molecular inhibition of myofibroblasts will mitigate scarring and contracture in a rabbit model of post-traumatic knee arthrofibrosis. The goal is to use small interfering RNAs as therapeutic agents, delivered through non-viral means. We have thus far established a baseline model of scar contracture across the knee joint in the rabbit hindleg that mimics a typical human clinical course: injury, followed by a period of fixation (8 weeks), followed by an extended period of release (16 weeks). We note marked contracture in the injured, operated joint, with none in the contralateral control hindlimb. Histologic and initial molecular analysis confirms dense intracapsular scar formation in the injured joints compared to contralateral control. These findings establish a significant baseline of injury against which our intervention may be measured.

15. SUBJECT TERMS

Scar, contracture, fibrosis, joint, extremity, trauma, myofibroblast, siRNA, minicircle

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	UU	9	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

Introduction

In recent conflicts, extremity injuries account for a very high percentage of all injuries suffered by military personnel, occurring in > 50% of all wounded warriors[1,2]. These injuries typically require operative debridement and fixation/immobilization; thus, a very high percentage of wounded warriors are at risk for the development of post-traumatic joint contracture/fibrosis. In multiple studies in both humans[3,4] and animals[5,6] it has been demonstrated that post-traumatic joint contractures demonstrate very high levels of myofibroblasts. Myofibroblasts are the cells responsible for exerting deformational forces during healing after injury, and for the deposition of the hyperabundant collagenous matrix characteristic of fibrosis. Myofibroblasts are marked by their unique expression of α-smooth muscle actin (α-SMA), which, together with myosin, provides the cells with their high contractility and motility. These cellular properties, writ large on a tissue scale, are responsible for the fibrotic, contracted phenotype seen after injury. The central idea for this proposal is to administer agents, specifically, small interfering RNAs (siRNAs) or nonviral vectors expressing siRNAs that will inhibit myofibroblast function, and thereby prevent the development of post-traumatic joint contracture. In order to test this hypothesis, it is first necessary to establish an animal model system which manifests joint contracture and fibrosis and which faithfully recapitulates the events leading to joint contracture in humans, that is, injury, fixation, followed by release. Once this model of elevated scar/contracture is validated, our molecular therapies may be examined against it to determine their effectiveness.

Keywords

Scar, contracture, fibrosis, joint, extremity, trauma, myofibroblast, siRNA, minicircle

Overall Project Summary

We have established the baseline rabbit injury protocol in which our proposed interventions are tested. This involves a complicated series of surgical maneuvers: first an arthrotomy is made, then the anterior and posterior cruciate ligaments are directly transected. The joint capsule is then further ruptured by hyperextension of the leg to a 135 degree angle. The hindleg is then placed into a position of maximal flexion with internal fixation using a buried Kirschner wire. After a period of 8 weeks, a second survival surgery is carried out to remove the K-wire. The animal is then allowed to recover for a further 16 weeks to see if any degree of joint mobility may be regained.

At the end of said 16 weeks animals are examined to determine the degree of contraction that obtains at the knee joint. In the first group of completed animals we found that the unaffected, unoperated hindlimb shows complete extension, as expected. In contrast, the injured, operated hindlimb maintains a significant scar contracture, also consistent with expected results. Pictures showing the difference between the two are shown below in Figure 1 below.

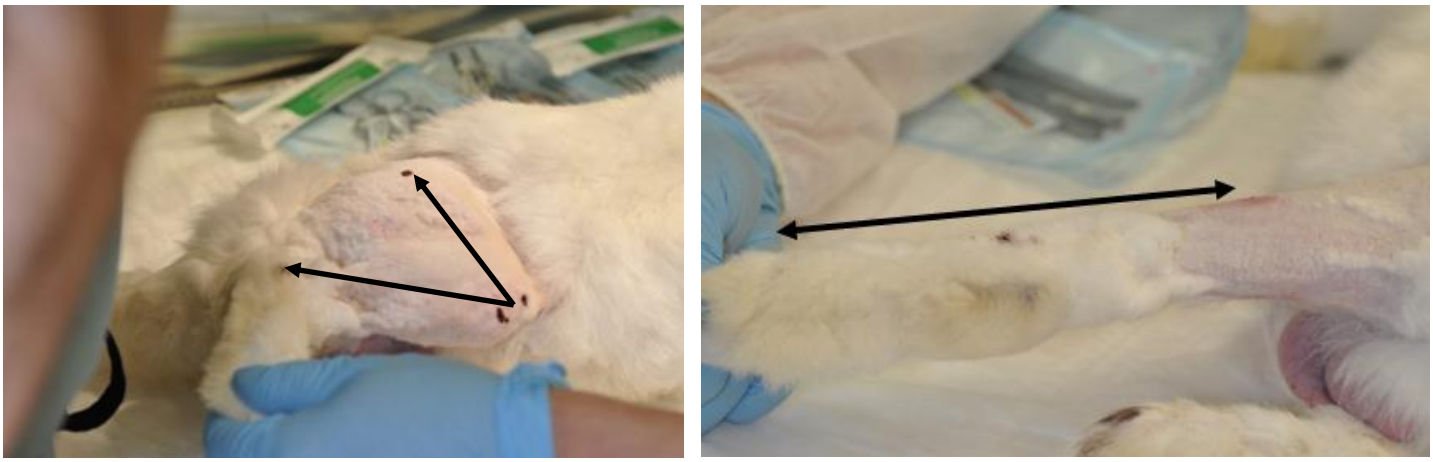

Figure 1: Photos of joint contracture after 24 weeks. (*left*) An operated hindlimb after 8 weeks fixation and then 16 weeks of release. The limb maintains an acute scar contracture. (*right*) The unoperated contralateral hindlimb of the same animal. Full extension is achieved.

Animals were then sacrificed and their joint/capsular tissues immediately harvested for histologic and molecular analysis. Operated knee joints showed dense fibrotic accumulations essentially obliterating the joint space. The contralateral unoperated joints showed essentially normal joint architecture, with normal appearing joint synovium which appeared to have some fatty component to its substratum as well. Samples from both were sent for hematoxylin and eosin as well as Masson's trichrome staining. Pictures of representative findings are shown in Figure 2 below.

Tissues were also stored in RNA later, then had total RNA extracted and characterized. Quantitative RT-PCR was carried out for α-smooth muscle actin (α-SMA) expression in scarred tissues versus the uninjured intracapsular ligamentous tissues of the unoperated hindlimb. Preliminary results are shown in Figure 3 below.

These results are all in accordance with expectations, specifically that injured limbs will heal with dense sclerotic scar formation and contracture. They give confidence of a significant pathological baseline against which our proposed molecular intervention can be measured.

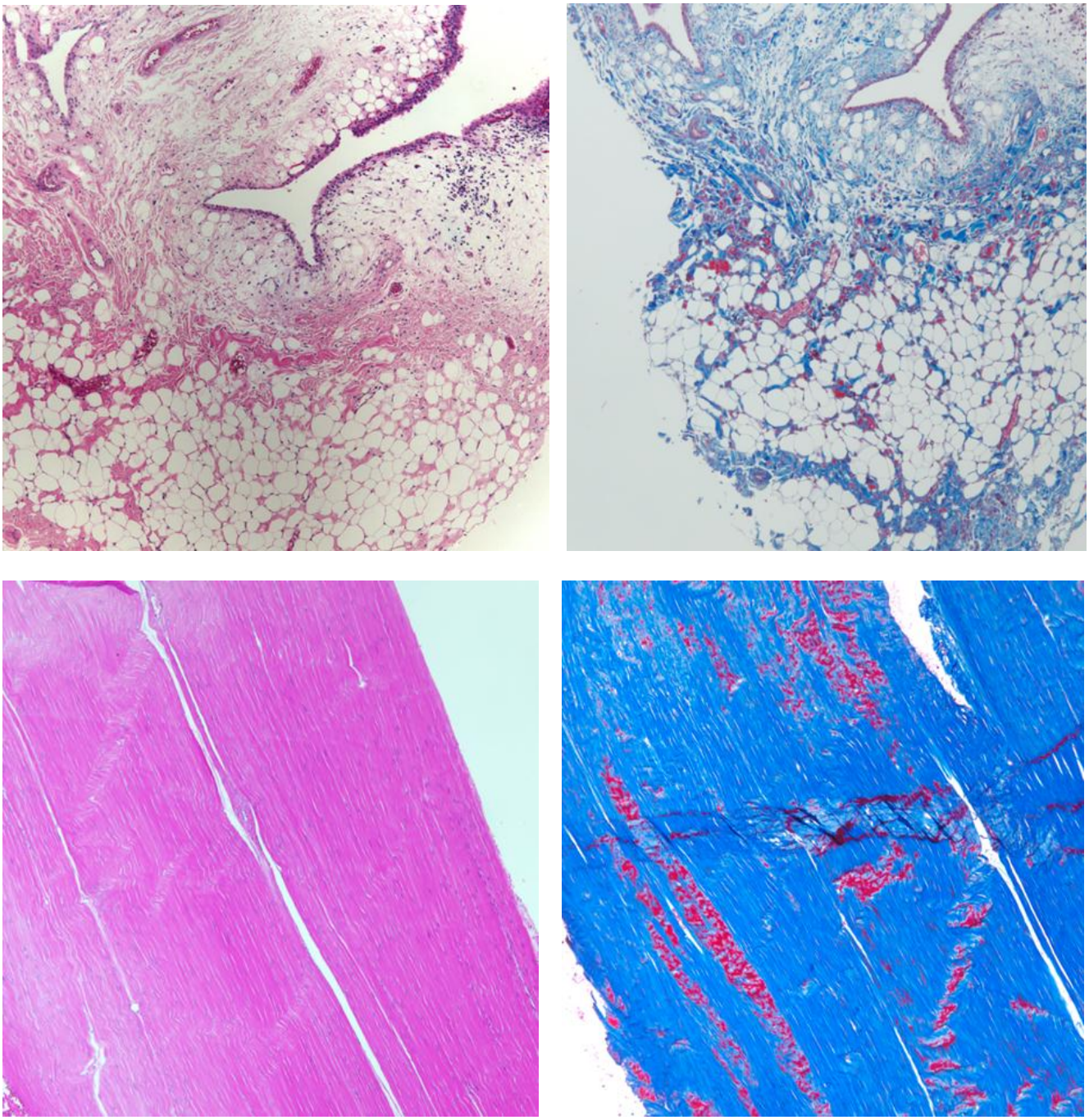

Figure 2. Histological examination of intracapsular tissue from unoperated and operated hindlimbs. (*top left*) H & E staining of uninjured, unoperated hindlimb tissue showing normal synovial and subsynovial architecture. (*top right*) Unoperated intracapsular tissue stained with Masson's trichrome. (*bottom left*) H & E staining of injured joint tissue. The normal tissue has been essentially completely replaced by scar. (*bottom right*) Masson's trichrome staining of injured joint tissue, again showing the dense collagen deposition of scar.

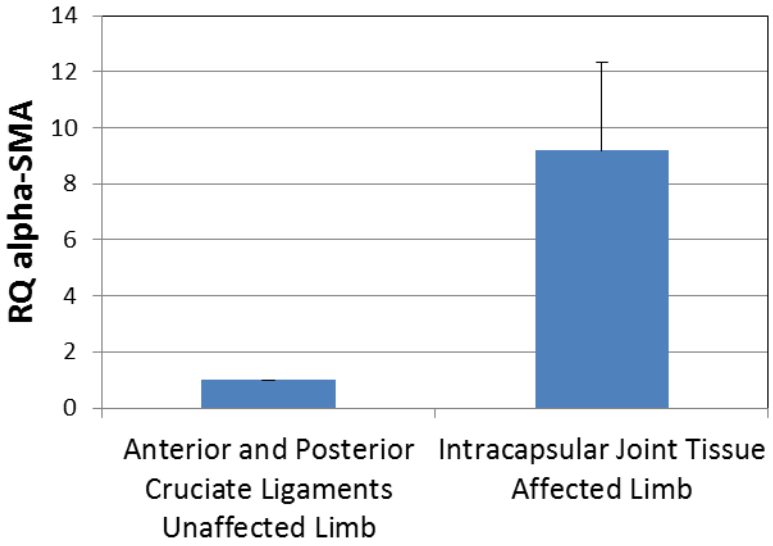

Figure 3. qRT-PCR determination of α-SMA in operated vs unoperated intracapsular tissue. Cruciate ligamentous tissue was assayed from unoperated limbs, versus dense scar tissue from operated limbs. Markedly elevated α-SMA was observed in the latter.

We have also made progress on the construction and testing of minicircle vectors for use in our studies. We have subcloned the RSV promoter/enhancer complex into the parent vector pMC-MCS2 and verified its position and orientation by confirmatory sequencing. We have subsequently added a 3' polyA signal sequence to increase message stability. This vector has now been transfected using ultrasound-assisted gene transfer into NIH 3T3 cells in vitro as a first attempt to examine its strength and durability of expression. The minicircle vector is able to drive expression of the luciferase reporter for a minimum of seven days, as shown in Figure 4 below.

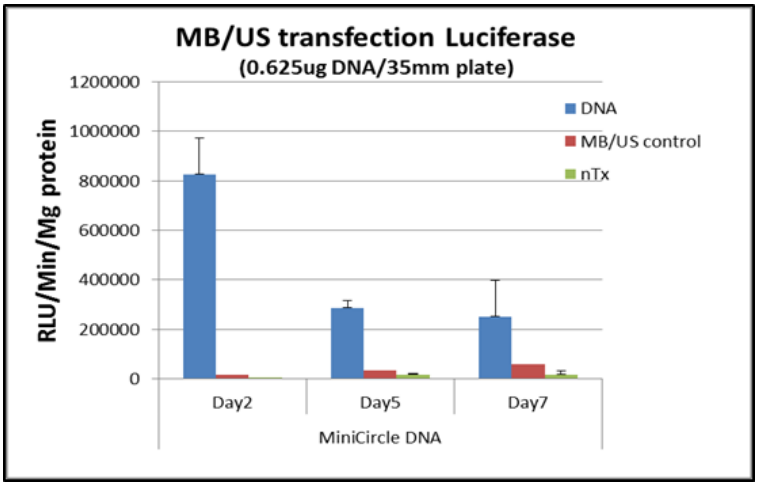

Figure 4. Luciferase reporter expression after ultrasound-assisted gene transfer into cells in vitro. Siginificant expression is seen when minicircle vector is used (DNA). Plasmid without reporter yields little expression (MB/US control). No transfection also gives little to no signal (nTx).

These are again encouraging preliminary results for the utility of our system.

Key Research Accomplishments

1. First demonstration that injury in this model of joint contracture yields a histological and molecular profile similar to that obtained in scirrhous cutaneous scar formation.
2. Successful demonstration of ultrasound-assisted gene transfer using a minicircle vector.

Conclusion

1. The animal model we have established offers an appropriately elevated level of scar and contracture against which molecular intervention can now be measured.

Publications, Abstracts and Presentations

None.

Inventions, Patents and Licenses

None.

Reportable Outcomes

None.

References

1. Peoples GE, Gerlinger T, Craig R, Burlingame B. Combat casualties in Afghanistan cared for by a single Forward Surgical Team during the initial phases of Operation Enduring Freedom. Mil Med. 2005 Jun;170(6):462-8.

2. Gondusky JS, Reiter MP. Protecting military convoys in Iraq: an examination of battle injuries sustained by a mechanized battalion during Operation Iraqi Freedom II. Mil Med. 2005 Jun;170(6):546-9.

3. Unterhauser FN, Bosch U, Zeichen J, Weiler A. Alpha-smooth muscle actin containing contractile fibroblastic cells in human knee arthrofibrosis tissue. Winner of the AGA-DonJoy Award 2003. Arch Orthop Trauma Surg. 2004 Nov;124(9):585-91.

4. Hildebrand KA, Zhang M, van Snellenberg W, King GJ, Hart DA. Myofibroblast numbers are elevated in human elbow capsules after trauma. Clin Orthop Relat Res. 2004 Feb;(419):189-97.

5. Hildebrand KA, Sutherland C, Zhang M. Rabbit knee model of post-traumatic joint contractures: the long-term natural history of motion loss and myofibroblasts. J Orthop Res. 2004 Mar;22(2):313-20.

6. Abdel MP, Morrey ME, Barlow JD, Kreofsky CR, An KN, Steinmann SP, Morrey BF, Sanchez-Sotelo J. Myofibroblast cells are preferentially expressed early in a rabbit model of joint contracture. J Orthop Res. 2012 May;30(5):713-9.